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14. ABSTRACT:
AIB1 which stands for "Amplified in Breast Cancer", codes for a protein that is a member of the steroid receptor coactivator (SRC) family. AIB1 is amplified in approximately 5-10% of breast cancers and the mRNA and protein overexpressed in >30% of breast cancers. AIB1 interacts with a superfamily of ligand activated nuclear receptors to potentiate transcriptional activity leading to upregulation of downstream target gene expression. An important finding was that an isoform of AIB1 (Δ3AIB1) is a significantly more effective coactivator of the estrogen receptor than AIB1 and is highly overexpressed in human breast cancer. Prior work in our lab showed that the downregulation of overall levels of AIB1 plus Δ3AIB1, using a regulatable AIB1 directed ribozyme, resulted in reduced tumor growth in vivo. Overall, these data indicate a major role for AIB1 and its isoform Δ3AIB1 in breast cancer development and growth. However the relative roles of AIB1 versus the more highly active Δ3AIB1 in phenotypic changes in the breast has not been determined. In this investigation, we are developing a method to use siRNA directed at Δ3AIB1 in order investigate its role in breast cancer and as a possible future therapeutic approach to breast cancer.

15. SUBJECT TERMS
Δ3AIB1, breast cancer

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INTRODUCTION

Of particular interest to breast cancer was the discovery that an area of chromosome 20q, known to be frequently amplified in breast cancer, harbored the gene for AIB1. AIB1 which stands for " **A**mplified **i**n **B**reast cancer" codes for a protein which is a member of the steroid receptor coactivator (SRC) family. AIB1 is amplified in approximately 5-10% of breast cancers and the mRNA and protein overexpressed in >30% of breast cancers. AIB1 interacts with a superfamily of ligand activated nuclear receptors including the estrogen receptor (ER) and progesterone receptor (PR) to potentiate transcriptional activity leading to upregulation of downstream target gene expression. An important finding was that an isoform of AIB1 (Δ3AIB1) is a significantly more effective coactivator of the estrogen receptor than AIB1 and is highly overexpressed in human breast cancer. Prior work in our lab showed that the downregulation of overall levels of AIB1 plus Δ3AIB1, using a regulatable AIB1 directed ribozyme, resulted in reduced tumor growth *in vivo*. Overall, these data indicate a major role for AIB1 and its isoform Δ3AIB1 in breast cancer development and growth. However the relative roles of AIB1 versus the more highly active Δ3AIB1 in phenotypic changes in the breast has not been determined. In this report I will summarize previous work from Dr. Mani and also the progress achieved in the past year.

BODY

Task 1: **Design small interfering siRNA molecules that specifically target the nuclear receptor coactivator isoform Δ3AIB1.**

In addition to developing siRNAs to target the nuclear receptor coactivator isoform Δ3AIB1, we are now in the process of designing and developing a lentiviral plasmid system to express short-haripin RNAs to target AIB1 and Δ3AIB1. (Figure 1) Currently, we have successfully applied a lentiviral system to target AIB1 with three of five of the short-hairpin RNAs we tried. Our results showed that we are able to knockdown protein levels of AIB1 in the human breast cancer cell line MDA-MB-231. (Figure 2) The lentiviral system was also able to knockdown protein levels of AIB1 in other breast cancer cell lines including the MCF-7 breast cancer cell line. This lentiviral system will serve as a framework to develop a lentiviral expressed short hairpin RNAs to target the Δ3AIB1 isoform.

NCOA3 (AIB1) shRNA-lentivirus

TRCN0000019699
CCGGGCCGCATTACTACAGGAGAAACTCGAGTTTCTCCTGTAGTAATGCGGCTTTTT
Clone ID: NM_006534.2-957s1c1
Accession Number(s): NM_181659.1, NM_006534.2

TRCN0000019700
CCGGCCATACATTTAATTGCCGTATCTCGAGATACGGCAATTAAATGTATGGTTTTT
Clone ID: NM_006534.2-781s1c1

TRCN0000019701
CCGGCCTCCGCAACAGTTTCCATATCTCGAGATATGGAAACTGTTGCGGAGGTTTTT
Clone ID: NM_006534.2-4106s1c1

TRCN0000019702
CCGGCCTCTACATCTGGAGGAGTATCTCGAGATACTCCTCCAGATGTAGAGGTTTTT
Clone ID: NM_006534.2-2187s1c1

TRCN0000019703
CCGGGCAGTCTATTCGTCCTCCATACTCGAGTATGGAGGACGAATAGACTGCTTTTT
Clone ID: NM_006534.2-2740s1c1

Figure 1: We obtained the above listed five sequences for short-hairpin RNAs to knockdown AIB1 protein levels from the RNAi Consortium. This database was developed by MIT and contains a lentiviral plasmid library that expresses short-hairpin RNAs to target 15,000 human genes (http://www.broad.mit.edu/genome_bio/trc/).

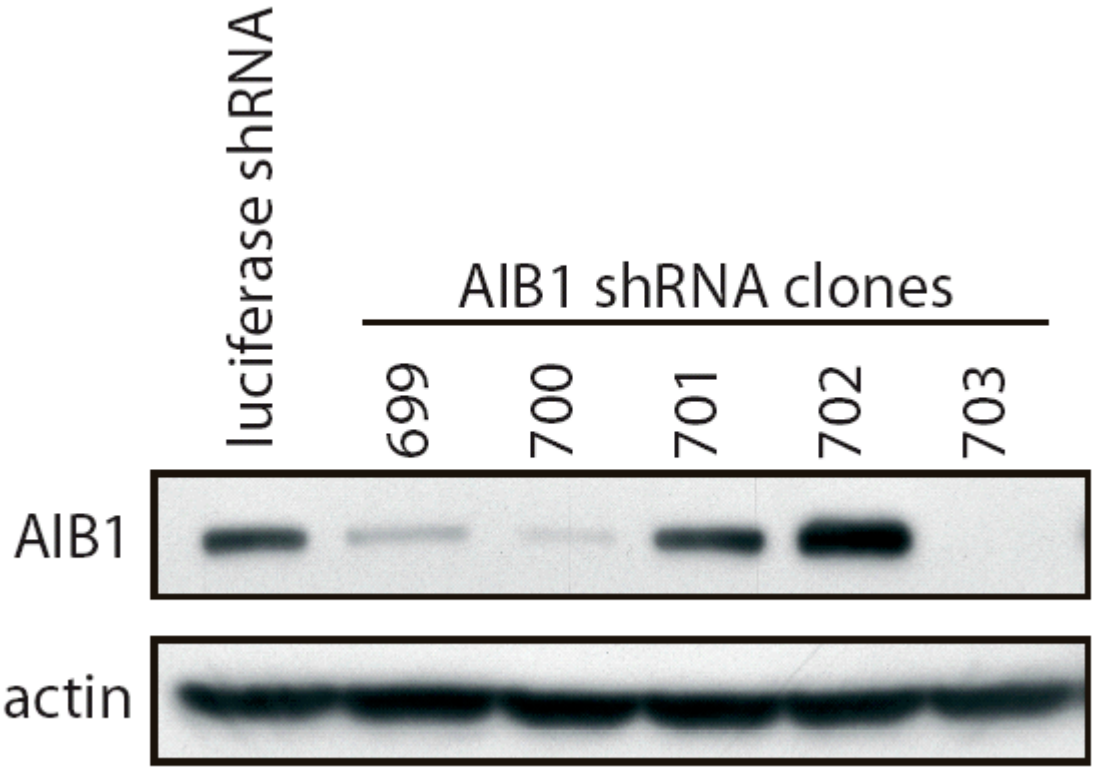

Figure 2: HEK293T cells were transfected with the individual shRNA vector, packaging vector, and envelope vector. Twenty four hours after plating, the cells were replaced with fresh media. This media or supernatant was then collected after twenty-four hours, filtered and then added to MDA-MB 231 cells. After twenty four hours, the MDA-MB 231 cells were harvested, a protein lysate was made and analyzed by western blot for AIB1 expression. Actin was used as a loading control.

Task 2: To determine if siRNA reduction of cellular levels of AIB1 of Δ3AIB1 can change the phenotype of breast cancer cell lines.

During ongoing studies of Δ3AIB1 in breast cancer, we have recently observed that AIB1 is degradaded in response to serum withdrawal and high cell density conditions in several immortalized cell lines. **We have further found that the Δ3AIB1 isoform is resistant to degradation in high cell density conditions**. (Figure 3) Recent data suggest that this is a proteasome related effect (see Mani et al in appendix). It would be interesting to target Δ3AIB1 by shRNA developed under Task 1 to see if it is possible to knockdown Δ3AIB1 protein levels in high cell density conditions. This result may hold important implications for the study we plan to do in this task where we will specifically target Δ3AIB1 isoform. It is interesting to note that another construct ΔN19 AIB1, which lacks the first 19 amino acids of the N terminus of AIB1 has also been shown to be resistant to proteasomal degradation[1]. In this paper they suggest that ΔN19 is resistant to degradation because it lacks a nuclear localization signal and that importation into the nucleus is vital for regulation by the proteasome and for its coactivator function. Δ3AIB1 may be resistant to degradation because it also lacks the nuclear localization sequence however, we and other groups have shown that Δ3AIB1 is a more potent coactivator than full length AIB1 and is overexpressed in breast cancer. I am currently investigating the localization and trafficking of Δ3AIB1 in relation to ΔN19AIB1 to see if this discrepancy can be resolved.

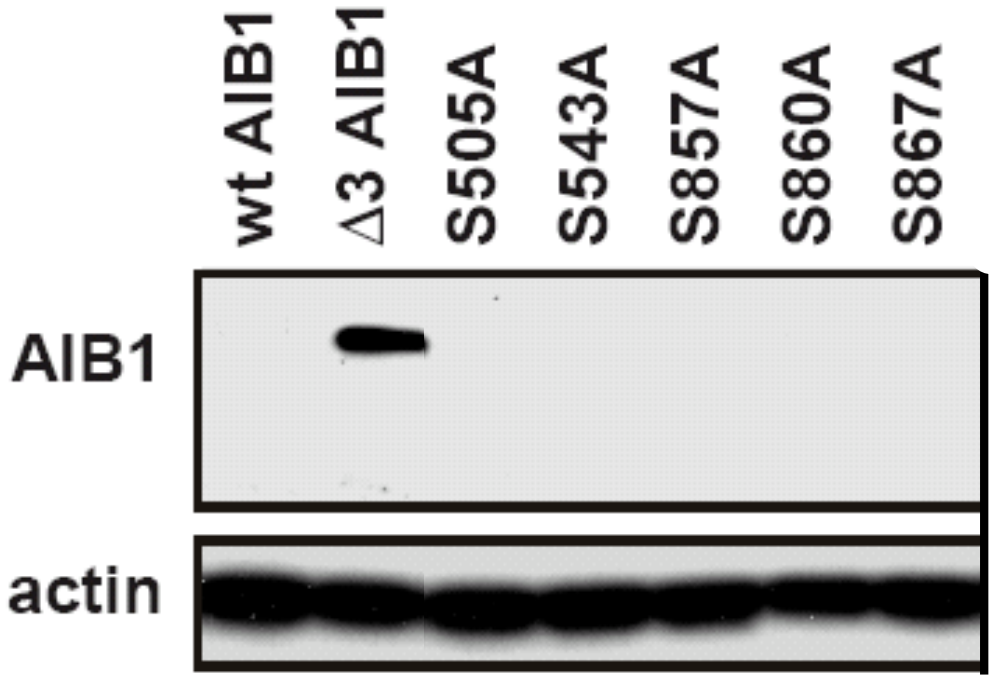

Figure 3: HEK293T cells were plated at 2.75x10^6 cells per 100mm tissue culture plate. Cells were transfected with wildtype AIB1, Δ3AIB1 isoform, or AIB1 serine phosphorylation mutants 24 hours after plating. Cells were harvested with RIPA buffer 24 hours after transfection and analyzed by Western blot for AIB1 expression. Results are representative of 2 separate experiments.

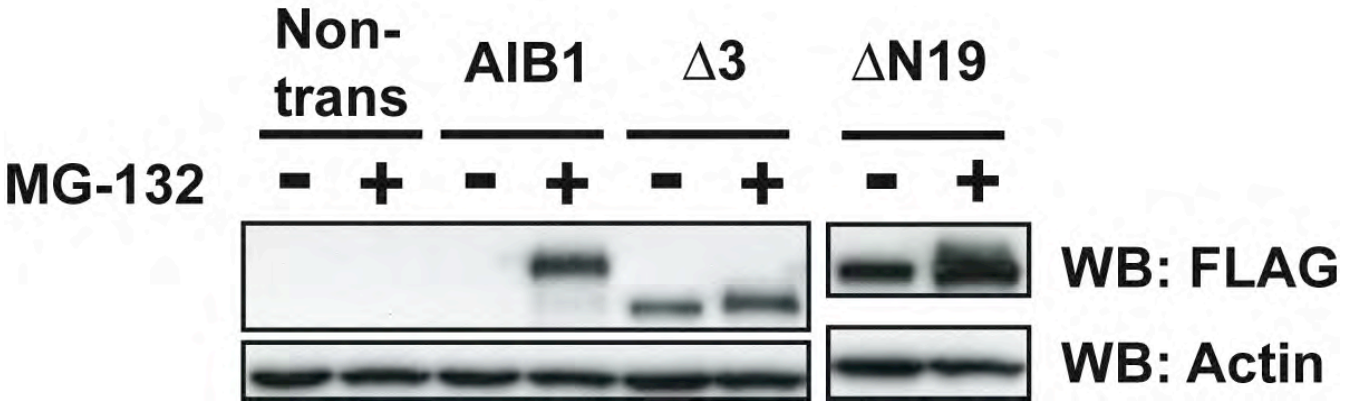

Figure 4: HEK293 cells were plated at 3.8x10^6 cells per 100mm poly d-lysine coated tissue culture plate. Cells were transfected with wildtype FLAG tagged AIB1, FLAG tagged Δ3AIB1 isoform, or FLAG tagged AIB1Δ1-19 24 hours after plating. Cells were treated with the proteasome inhibitor MG-132 or an equal volume of DMSO overnight 24 hours post transfection. Cell lysates were then prepared in 1% NP-40 lysis buffer and Western blotted for FLAG and actin. Results are representative of two separate experiments.

Task 3: **To determine if Δ3AIB1 siRNA is effective *in vivo*.**

We have not formally begun this task since we are awaiting the development of the Δ3AIB1 siRNA that specifically knocks down Δ3AIB1 expression. The recent observation that we made showing that the Δ3AIB1 isoform is resistant to density dependent proteasomal degradation to which the wild-type AIB1 is susceptible is an intriguing and novel finding. We speculate that resistance to degradation displayed by the Δ3AIB1 isoform in a high cell density condition might provide a mechanistic explanation for the finding by Reiter et al that the isoform's ability to promote transcription mediated by the estrogen or progesterone receptors was significantly greater than that of the full-length protein.[2]

KEY RESEARCH ACCOMPLISHMENTS

Development of a lentiviral plasmid system to express short-haripin RNAs to target AIB1.

Defined resistance of Δ3AIB1 to proteasomal degradation.

REPORTABLE OUTCOMES

"E6AP Mediates Regulated Proteasomal Degradation of the Nuclear Receptor Coactivator Amplified in Breast Cancer 1 in Immortalized Cells." **Aparna Mani**, Annabell S Oh, Emma T Bowden, Tyler Lahusen, Kevin L Lorick, Allan M Weissman, Richard Schlegel, Anton Wellstein, and Anna T. Riegel, *Cancer Research* 2006 Sep 1;66(17):8680-6.

"Δ3AIB1 Localization Illustrates a Paradigm for P160 Coactivator Function and Regulation." Annabell S Oh, Chris Chien, Tyler Lahusen, Anton Wellstein, and Anna T. Riegel, *Manuscript in preparation*.

CONCLUSIONS

The work done to date as outlined in this report illustrates that it is possible to specifically target AIB1 using a short-haripin RNAs expressed through a lentiviral system. This gives us an initial framework from which we can design and develop short-haripin RNAs to specifically target Δ3AIB1. In addition, we plan to continue studies on the intriguing finding the isoform Δ3AIB1 is resistant to proteasomal degradation compared to wild-type AIB1. We are currently investigating the localization and coactivator function of Δ3AIB1 in relation to ΔN19AIB1 to try to resolve the discrepancy that Δ3AIB1 is a potent coactivator though it lacks the nuclear localization signal to enter the nucleus.

REFERENCES

1. Li et al. 2007, *Mol Cell Bio.* V27 No.4:1296-1308.

2. Reiter et al. 2001, *J Biol Chem.* V276:39736-41.

E6AP Mediates Regulated Proteasomal Degradation of the Nuclear Receptor Coactivator Amplified in Breast Cancer 1 in Immortalized Cells

Aparna Mani,[1] Annabell S. Oh,[1] Emma T. Bowden,[1] Tyler Lahusen,[1] Kevin L. Lorick,[2] Allan M. Weissman,[2] Richard Schlegel,[1] Anton Wellstein,[1] and Anna T. Riegel[1]

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Abstract

The steroid receptor coactivator oncogene, amplified in breast cancer 1 (AIB1; also known as ACTR/RAC-3/TRAM-1/SRC-3/p/CIP), is amplified and overexpressed in a variety of epithelial tumors. AIB1 has been reported to have roles in both steroid-dependent and steroid-independent transcription during tumor progression. In this report, we describe that the cellular levels of AIB1 are controlled through regulated proteasomal degradation. We found that serum withdrawal or growth in high cell density caused rapid degradation of AIB1 protein, but not mRNA, in immortalized cell lines. Proteasome inhibitors prevented this process, and high molecular weight ubiquitylated species of AIB1 were detected. Nuclear export was required for proteasomal degradation of AIB1 and involved the ubiquitin ligase, E6AP. AIB1/E6AP complexes were detected in cellular extracts, and reduction of cellular E6AP levels with E6AP short interfering RNA prevented proteasomal degradation of AIB1. Conversely, overexpression of E6AP promoted AIB1 degradation. The COOH terminus of AIB1 interacted with E6AP *in vitro* and deletion of this region in AIB1 rendered it resistant to degradation in cells. From our results, we propose a model whereby signals promoted by changes in the cellular milieu initiate E6AP-mediated proteasomal degradation of AIB1 and thus contribute to the control of steady-state levels of this protein. (Cancer Res 2006; 66(17): 8680-6)

Introduction

Amplified in breast cancer 1 (AIB1) is the only member of the nuclear receptor coactivator family that is amplified (on chromosome 20q) and overexpressed in a wide variety of human epithelial tumors (1–6). AIB1 is also known as ACTR/RAC-3/TRAM-1/SRC-3/p/CIP; refs. 7–10. AIB1 is known to drive preneoplastic and neoplastic changes in mice (11, 12) and has been defined as an oncogene (12). Loss of AIB1 can abrogate Ha-*ras*-induced tumorigenesis in mice (13). Furthermore, high levels of AIB1 are associated with overexpression of the oncogene HER-2/*neu* (14, 15). In some tumors, AIB1 is rate limiting for steroid signaling (16) but it is also important for insulin-like growth factor (IGF)-I–induced oncogenic changes and signaling both in mice (17) and in tumor cell lines (18). Irrespective of the precise role of AIB1 in oncogenesis, it is probable that the cellular levels of AIB1, like many molecules involved in integrating survival signaling, would normally be tightly controlled. We have determined previously that estrogen can regulate AIB1 levels in breast cancer cells (19), but little is known about the control of cellular levels of AIB1 protein in other epithelial cell types.

A common mechanism of control of steady-state concentrations of cellular protein levels is through regulated proteasomal degradation. The ubiquitin-proteasome pathway consists of an enzymatic cascade, which ubiquitylates cellular proteins targeting them for proteasomal degradation. The ubiquitin-activating enzyme, E1, binds ubiquitin through a thioester linkage in an ATP-dependent step (20, 21). The activated ubiquitin is then transferred to the ubiquitin-conjugating enzyme, E2. E2 works in conjunction with the ubiquitin ligase enzyme, E3, which is responsible for conferring substrate specificity (22). E3 mediates the transfer of ubiquitin to the target protein. The polyubiquitylated target protein undergoes proteolytic cleavage by the proteasome (23–25). Although, it has been shown that steady-state levels of AIB1 are increased when cells are treated with a proteasome inhibitor (26, 27), the regulation of AIB1 proteasomal degradation has not been described previously.

In this study, we challenged different tumor cell lines by serum deprivation or growth at high cell density to reveal the contribution and mechanism of proteasomal control of steady-state levels of AIB1. We observed that there was a rapid, nuclear export–dependent down-regulation of AIB1 protein levels. We hypothesized that this could be through a regulated, ubiquitin-mediated, proteasomal mechanism and sought to determine the identity of the ubiquitin ligase that would recognize AIB1 for regulated degradation. We now report that the ubiquitin ligase E6AP plays a major role in the control of the regulated degradation of AIB1.

Materials and Methods

Cell lines and reagents. Serum withdrawal experiments were conducted as follows for COLO 357 PL (L3.6pl obtained from Dr. Kim Jessup, Georgetown University, Washington, DC) pancreatic cancer cells (28), MCF-7 breast cancer cells [American Type Culture Collection (ATCC), Manassas, VA], and ME180 squamous carcinoma cells (ATCC). Cells were plated at a low density overnight in full serum. The cells were washed twice with improved minimal essential medium (IMEM) and medium was replaced with serum-free IMEM+/− inhibitor or full serum medium 24 hours after plating. Protein expression was observed over the next 24 to 36 hours. For HEK293T cells (ATCC), an AIB1 expression vector was transfected before plating for the serum withdrawal experiment as described above. To determine the effect of cell density on AIB1 regulation, COLO 357 PL cells were plated at a density of 4×10^6 (high density) or 1×10^6 (low density) per 100-mm tissue culture dish in full serum. Whole-cell lysates were prepared in NP40 lysis buffer [1% NP40, 20 mmol/L Tris-HCl, 150 mmol/L

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NaCl, 2.5 mmol/L EDTA, 10 mmol/L NaF, 10 mmol/L NaPPi, Roche complete protease inhibitor mixture (Roche Applied Biosciences, Indianapolis, IN)]. Chemical inhibitors were purchased from Calbiochem (Darmstadt, Germany) and used at the following concentrations: 5 μmol/L MG132, 10 μmol/L ALLN, 10 μmol/L ALLM, 10 μmol/L lactacystin, and 75 μmol/L leptomycin B.

Plasmids and antibodies. The pcDNA6/V5-His-AIB1 (1-809) construct was made by restriction digest of pcDNA3-AIB1/ACTR (from Dr. H.W. Chen, University of California Davis, Sacramento, CA) with HindIII in the multiple cloning site of the vector and *Xba*I in the AIB1 cDNA. This fragment was subcloned subsequently into pcDNA6/V5-His digested with HindIII and *Xba*I. The myc-tagged mouse E6AP expression vector was constructed as described previously (29). The human E6AP expression vector was obtained from Dr. Peter Howley (Harvard University, Boston, MA; ref. 30). The HA-ubiquitin plasmid was obtained from Dr. Chenguang Wang (Thomas Jefferson University, Philadelphia, PA). The antibodies used were obtained from the following companies: AIB1 (BD Transduction Laboratories, Mississauga, Ontario, Canada), actin (Millipore, Billerica, MA), ubiquitin (Invitrogen, Carlsbad, CA), HA (Roche), histone deacetylase 1 (HDAC1; Millipore), SNF2β/BRG1 (Millipore), Myc (Sigma, St. Louis, MO), FLAG M2 (Sigma), mouse IgG (Millipore), and V5 (Invitrogen). The E6AP polyclonal antibody was obtained from Dr. Norman Maitland (University of York, York, United Kingdom; ref. 31).

Short interfering RNA transfections. COLO 357 PL or ME180 cells were plated in full serum overnight. LipofectAMINE 2000 reagent (Invitrogen) was used to transfect cells 24 hours after plating with control short interfering RNA (siRNA), E6APA siRNA, or E6APB siRNA. Cell lysates were harvested and analyzed by Western blot 48 hours after transfection. E6AP DNA target sequences E6APA and E6APB as described in ref. 32 were used to generate sense and antisense RNA sequences (Qiagen, Valencia, CA). The control siRNA (Cx) was generated as described (18).

Western blot, immunoprecipitation, and coimmunoprecipitation analyses. For detection of high molecular weight (HMW) ubiquitylated forms of AIB1, COLO 357 PL cells were plated as described for serum withdrawal experiments and treated with DMSO or ALLN. Cells were lysed with NP40 lysis buffer, 10 hours after serum withdrawal, and immunoprecipitated with γ-bind G-Sepharose beads (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) and 1 μg AIB1 monoclonal antibody. The immunoprecipitated proteins were separated by SDS-PAGE and transferred to polyvinylidene membrane, which was probed with an anti-AIB1 monoclonal antibody or anti-ubiquitin antibody.

For HA-ubiquitin coimmunoprecipitation experiments, MCF-7 cells were transfected with HA-tagged ubiquitin. Cells were washed twice with IMEM and replaced with full serum medium or serum-free IMEM in the presence of ALLN or DMSO 8 hours after transfection. Cell lysates were harvested 24 hours after transfection with radioimmunoprecipitation assay buffer (RIPA; 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 40 mmol/L glycerol 2-phosphate, 0.25% sodium deoxycholate, 1% NP40, 50 mmol/L NaF, 20 mmol/L NaPPi, 1 mmol/L EGTA) and immunoprecipitated with AIB1 antibody as described above. The immunoprecipitated proteins were analyzed as described above by Western blot with anti-HA antibody.

For coimmunoprecipitation experiments, HEK293T cells were transfected using Fugene 6 reagent (Roche) with equal amounts of expression vectors for FLAG-tagged AIB1 or V5-tagged AIB1 (1-809) and myc-tagged E6AP. MCF-7 cells were transfected with myc-tagged E6AP or pcDNA3 vector. Medium was replaced with serum-free IMEM containing ALLN 9 hours after transfection. For HEK293T cells, cell lysates were harvested 24 hours after transfection with RIPA and immunoprecipitated with FLAG M2 antibody, mouse IgG, or V5 antibody. For MCF-7 cells, cell lysates were harvested 24 hours after transfection with RIPA and immunoprecipitated with myc antibody–conjugated agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA).

For Western blot analysis, HEK293T cells were plated at high density and transfected with expression vectors for FLAG-tagged AIB1 full-length or V5-tagged truncation mutant AIB1 (1-809). For overexpression study, HEK293T cells were cotransfected with E6AP plasmid or pcDNA3 vector and FLAG-tagged AIB1 plasmid. Cells were washed twice with IMEM and replaced with serum-free IMEM 8 hours after transfection. Cell lysates were harvested with RIPA 48 hours after transfection and analyzed by Western blot.

Immunofluorescence. COLO 357 PL cells were plated onto glass coverslips, and the serum deprivation experiment was done as described. The glass slides were fixed before the medium change (0 hour) and 24 hours after the medium change with 3.7% paraformaldehyde in PBS for 20 minutes. The cells were then permeabilized by treatment with 0.1% Triton X-100 in PBS for 5 minutes, stained with an AIB1 monoclonal antibody, and analyzed by confocal microscopy.

Quantitative reverse transcription-PCR analysis. Total cellular RNA was harvested with RNA signal transducers and activators of transcription (STAT) reagent before the medium change (0 hour) and at 10 and 24 hours after serum withdrawal and analyzed by real-time PCR according to the protocol described (18). Samples were run in duplicate and were normalized relative to glyceraldehyde-3-phosphate dehydrogenase. Primers and probe were made as described (18).

***In vitro* glutathione-*S*-transferase-binding assay.** All glutathione *S*-transferase (GST)–AIB1 (RAC3) fusion constructs were obtained from Dr. J.D. Chen (University of New Jersey, Piscataway, NJ; ref. 33). GST-tagged AIB1 fragments were purified and immobilized on glutathione sepharose beads. The beads were then incubated with MCF-7 whole-cell lysate overnight. The beads were washed five times with NP40 lysis buffer, resuspended in 30 μL 4× SDS sample buffer, and boiled. The bound E6AP was analyzed by Western blot with an E6AP antibody. To determine total expressed GST protein, equal aliquots of purified GST fragments were resolved on a gel, transferred to a polyvinylidene difluoride (PVDF) membrane, and analyzed by Western blot with a GST antibody (1:2,000; Santa Cruz Biotechnology).

Results

Regulated proteasomal degradation of AIB1/ACTR. To understand whether signaling events could alter AIB1 levels, we monitored the effect of serum deprivation on AIB1 mRNA and protein levels. We initially conducted these studies in metastatic pancreatic adenocarcinoma COLO 357 PL cells (28). We observed that serum withdrawal from COLO 357 PL cells resulted in a significant decrease (up to 70-90% at 24 hours) in AIB1 protein levels (Fig. 1*A*). This reduction in protein levels was consistently observed at 10 to 12 hours after serum withdrawal (Fig. 1*A*) but could often be observed as early as 6 hours after serum withdrawal (data not shown). In contrast, cells maintained in full serum showed no change or an increase in AIB1 levels during the same time (Fig. 1*A*). During serum withdrawal, the drop in AIB1 protein expression was not due to transcriptional repression of the *AIB1* gene because mRNA expression in the COLO 357 PL cells, measured by real-time PCR, was unchanged (Fig. 1*B*). These data suggested the possibility that there was an active protein degradation process occurring that was regulating the levels of AIB1 protein in response to serum withdrawal in these cells.

The ubiquitin proteasome pathway represents one mechanism in the cell for the active degradation of proteins. The ubiquitin proteasome degradation pathway is composed of ubiquitin, a three-enzyme ubiquitylation complex, the intracellular target proteins, and the proteasome that is the organelle of protein degradation. A protein that is destined for degradation by this process is first conjugated to a polyubiquitin chain, which then targets it to the proteasome for active degradation. Inhibition of the proteasome by chemical inhibitors results in the accumulation of the otherwise degraded protein. To determine whether AIB1 is degraded by the ubiquitin proteasome pathway in response to serum withdrawal, COLO 357 PL cells were subjected to serum withdrawal in the presence of various proteasome inhibitors, including MG132 or lactacystin, versus DMSO vehicle control and the peptide aldehyde proteasome inhibitor ALLN versus a related protease inhibitor

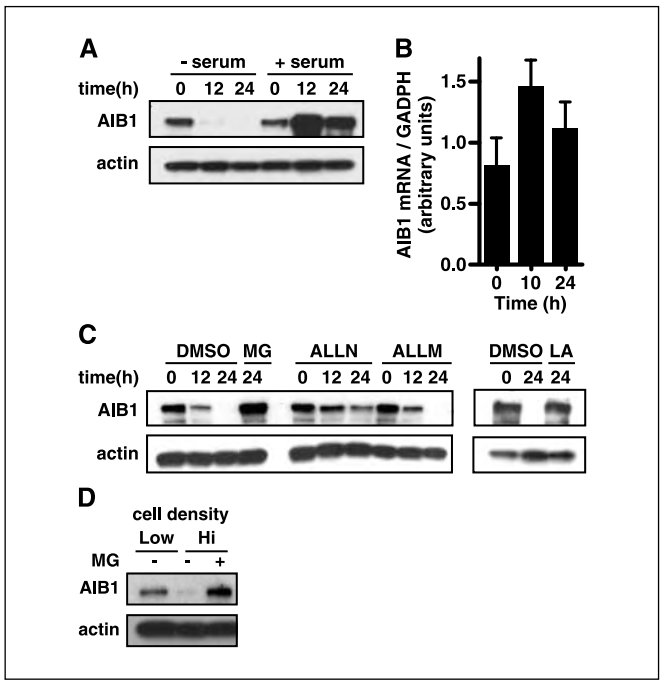

Figure 1. Serum deprivation leads to ubiquitin-mediated proteasomal degradation of AIB1. *A,* COLO 357 PL cells were serum deprived or left in full serum, and AIB1 protein expression was analyzed by Western blot before the medium change (0 hour) and at 12 and 24 hours after the medium change. *B,* AIB1 mRNA expression was quantified by real-time PCR at 0, 10, and 24 hours after serum withdrawal. *C,* effect of proteasome inhibitors on AIB1 expression assayed by Western blot of whole-cell extracts. COLO 357 PL cells were serum deprived and treated with DMSO and ALLM as negative controls or with the proteasome inhibitors MG132 (*MG*), ALLN, and lactacystin (*LA*). *D,* high cell density conditions lead to proteasomal degradation of AIB1. COLO 357 PL cells were plated in full serum at low density (1 million per 10-cm plate) and high density (4 million per 10-cm plate). Plates at high density were treated with MG132 proteasome inhibitor or DMSO 8 hours after plating. AIB1 protein expression was analyzed by Western blot 24 hours after plating.

control ALLM (Fig. 1*C*). Anti-AIB1 Western blots showed that the proteasome inhibitors were able to prevent the serum withdrawal–induced degradation of AIB1 protein compared with the control treatments. In addition, we examined other conditions, such as growth at high cell density (Fig. 1*D*). Similar to serum deprivation, we found a down-regulation of AIB1 protein in cells grown to high density and show that this involves the 26S proteasome because it is prevented by treatment with MG132 (Fig. 1*D*).

To show that AIB1 is directly ubiquitylated in response to serum withdrawal, we immunoprecipitated AIB1 from serum-starved COLO 357 PL cells treated with either the proteasome inhibitor ALLN or DMSO vehicle control and detected HMW, ubiquitylated forms of AIB1 by immunoblot (Fig. 2*A*). Only when cells were treated with the proteasome inhibitor were a significant amount of HMW AIB1-ubiquitin conjugates detected by Western blot (Fig. 2*A*). Furthermore, after serum withdrawal, transfected HA-tagged ubiquitin was able to coimmunoprecipitate with endogenous AIB1 in the presence of a proteasome inhibitor in MCF-7 cells (Fig. 2*B*). Ubiquitylation of AIB1 is enhanced with serum withdrawal and in the presence of proteasome inhibitor (Fig. 2*B*). Taken together, these data show that AIB1 is ubiquitylated and proteasomally degraded in response to serum withdrawal or high cell density. The down-regulation of the AIB1 protein in response to these growth conditions and its inhibition by proteasome inhibitors is not restricted to COLO 357 PL or MCF-7 cells because it was also observed in other cell lines, such as the squamous carcinoma cell line ME180 (Fig. 2*C*) and with exogenously transfected AIB1 in HEK 293T cells (Fig. 2*C*). Overall, these data indicate that changes in the extracellular milieu can induce rapid degradation of AIB1 and this phenomenon is observed in several epithelial cancer cell lines.

Proteasomal degradation of AIB1 requires nuclear export of AIB1. AIB1 is found in the nuclear and cytoplasmic compartments of cancer cells (34) and its movement between the cytoplasm and nucleus is dependent on growth factor signaling (34). The bulk of the AIB1 in the COLO 357 PL cells is located in the nucleus before serum withdrawal (Fig. 3*A*). However, low levels of cytoplasmic AIB1 are also detected (Fig. 3*A, inset*). After serum withdrawal for 24 hours, a significant reduction in AIB1 protein staining in the nucleus and the cytoplasm was observed and this was blocked by treatment with MG132 relative to the DMSO control (Fig. 3*A*).

Degradation of proteins by the 26S proteasome can occur in both cytoplasmic and nuclear compartments and we next determined whether AIB1 degradation required nuclear export. To investigate this, we used an antibiotic agent leptomycin B, which acts as a potent inhibitor of CRM-1/exportin 1-mediated active nuclear export (35, 36). COLO 357 PL cells were plated in full serum and pretreated with leptomycin B for 2 hours before serum withdrawal. Western blot analysis of AIB1 protein expression revealed that inhibition of nuclear export with leptomycin B prevented the serum withdrawal–induced degradation of AIB1 (Fig. 3*B*). The immunofluorescence data coupled with the leptomycin B study suggested that, before serum withdrawal, the bulk of the AIB1 protein is localized in the nucleus of the COLO 357 PL

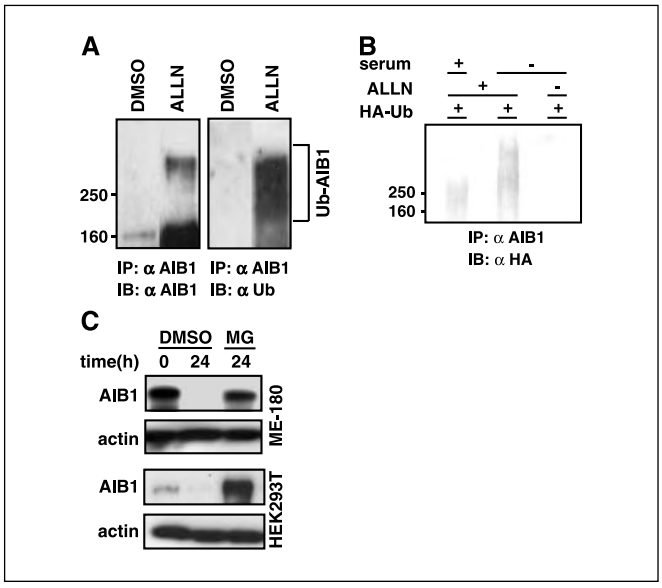

Figure 2. *A,* HMW ubiquitylated forms of AIB1 are observed after serum withdrawal. COLO 357 PL cell lysates were immunoprecipitated (*IP*) 10 hours after serum withdrawal with AIB1 antibody. The precipitated proteins were analyzed by Western blot (*IB*) with anti-AIB1 or anti-ubiquitin antibodies. *B,* transfected HA-tagged ubiquitin coimmunoprecipitates with endogenous AIB1. MCF-7 cells transfected with HA-tagged ubiquitin were serum deprived or left in full serum and treated with DMSO or proteasome inhibitor ALLN. Cell lysates were immunoprecipitated 12 hours after the medium change with AIB1 antibody. The precipitated proteins were analyzed by Western blot with anti-HA antibody. *C,* ME180 human squamous carcinoma or HEK293T cells transfected with AIB1 expression vector also exhibit serum withdrawal–induced proteasomal degradation of AIB1. ME180 or HEK293T cells were serum deprived and treated with DMSO or MG132. AIB1 expression was analyzed by Western blot before the medium change (0 hour) and at 24 hours after the medium change and treatment.

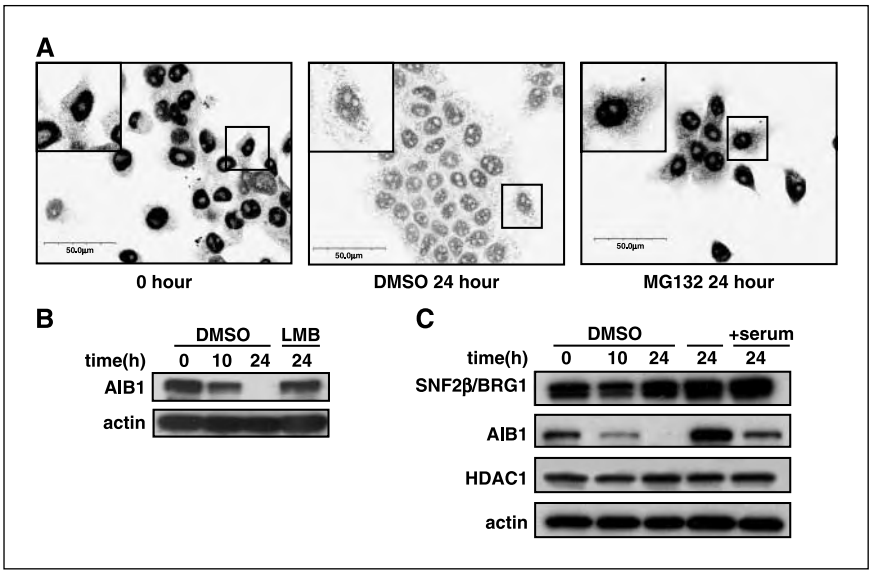

Figure 3. Proteasomal degradation of AIB1 occurs in the nuclear and cytoplasmic compartments and is dependent on nuclear export. *A,* immunofluorescence of COLO 357 PL cells before serum withdrawal (0 hour) and at 24 hours after serum withdrawal in the presence of DMSO vehicle or MG132. *Inset,* cytoplasmic and nuclear AIB1. *B,* treatment with the nuclear export inhibitor leptomycin B (*LMB*) prevents the serum withdrawal induced loss of AIB1. *C,* COLO 357 PL cells were replaced with serum-free IMEM in the presence or absence of MG132 or full serum medium 24 hours after plating. HDAC1 and SNF2β/BRG1 protein expression were observed before (0 hour) and 10 and 24 hours after the medium change Western blot analysis.

cells and that serum withdrawal leads to translocation of the nuclear AIB1 to the cytoplasm where it is rapidly degraded by the proteasome. Because serum withdrawal impinges on several different cellular signaling pathways, it was possible that the degradation of AIB1 was part of a generalized degradation of nuclear proteins after serum withdrawal in these cells. However, examination of other nuclear proteins involved in transcription complexes, localized in close contact with chromatin and known to be ubiquitylated, such as HDAC1 (37) or components of the SWI/SNF complex (SNF2β/BRG1), was unaffected by serum withdrawal (Fig. 3*C*), suggesting that the activation of proteasomal degradation of AIB1 regulated by serum withdrawal is relatively selective.

The ubiquitin ligase, E6AP, is involved in proteasomal degradation of AIB1. In an effort to delineate the mechanism by which AIB1 protein is regulated by the proteasome, we aimed to identify the potential ubiquitin ligase that may be involved in AIB1 degradation. Ubiquitin ligases are the components of the ubiquitin enzymatic cascade that confer substrate specificity (22). E6AP was originally identified as the ubiquitin ligase that interacts with the human papilloma type 16 and 18 E6 protein to mediate the degradation of the tumor suppressor p53 (38). A previous report has also shown that E6AP can act as a nuclear coactivator and potentiate the transcriptional activity of several steroid hormone receptors, including the estrogen receptor (39). We decided to primarily focus on E6AP as the putative ubiquitin ligase involved in the degradation of AIB1 because Shao et al. (40) have shown previously a direct interaction between AIB1 and E6AP. To examine if there was a direct interaction between AIB1 and E6AP in the cells used in this study, we did a coimmunoprecipitation assay in serum-deprived cells transfected with both myc-tagged E6AP and FLAG-tagged AIB1 (Fig. 4*A*) or with myc-tagged E6AP and endogenous AIB1 (Fig. 4*B*). We observed that there was a direct interaction between these proteins in these cells (Fig. 4*A* and *B*). Next to answer the question of whether E6AP is involved in the induced degradation of AIB1, we inhibited the expression of E6AP by siRNA and examined whether loss of E6AP would prevent proteasomal degradation of AIB1. We found that E6AP knockdown with two siRNAs (32) directed at different domains of E6AP both prevented the degradation of AIB1 protein in COLO 357 PL and in ME 180 human squamous carcinoma cells (E6AP is the upper arrowed band with a molecular weight (MW) of 100 kDa; Fig. 4*C* and *D*). Conversely, overexpression of transfected E6AP enhanced the degradation of cotransfected FLAG-tagged AIB1 (Fig. 4*E*). Overall, these results showed that AIB1 is in a complex with E6AP *in vivo* and suggest that formation of the complex is related to regulated degradation of AIB1.

To determine the region of AIB1, which is required for interaction with E6AP, we examined the interaction of GST-labeled fragments of AIB1 with E6AP (Fig. 5*A*). For this assay, we made cellular extracts from cells grown in full serum and determined the interaction of E6AP in these extracts with the AIB1 fragments fused to GST. The major interacting fragment was from 723-1,034 of AIB1 (Fig. 5*A*). These results suggested that the COOH terminus of AIB1 distal to amino acid 723 is required for the AIB1 interaction with E6AP. Although the levels of GST protein fragment produced from the vectors for regions 613-752 and 1,017-1,417 were less than the other fragments, we have observed that the 1,017-1,417 fragment prepared under these exact conditions is able to fully interact with CBP (data not shown). One prediction from this data is that removal of the E6AP COOH-terminal binding region from AIB1 will render AIB1 insensitive to proteasomal degradation. To determine this, we used an AIB1 expression construct from amino acids 1-809, which lacked the COOH-terminal amino acids from 810-1,417. This construct harbored a COOH-terminal V5 tag and was unable to interact with E6AP after transfection into HEK293T cells (Fig. 5*B*), although the transfected full-length AIB1 was able to coimmunoprecipitate efficiently with E6AP (Fig. 5*B*). When this construct was transfected into HEK293T cells, at 24 hours after transfection, in high confluence conditions, the AIB1 1-809 proteins were easily detected, whereas the transfected full-length control AIB1 was degraded fully (Fig. 5*C, left*). This was not due to transfection problems because, at low-density conditions, the transfected full-length AIB1 plasmid and AIB1 1-809 mutants produced equal amounts of protein (Fig. 5*C, right*). Thus, loss of the COOH terminus of AIB1 renders AIB1 resistant to regulated proteasomal degradation.

Discussion

This is the first report, to our knowledge, of the requirement for the ubiquitin ligase E6AP for the regulated degradation of a nuclear receptor coactivator. E6AP has also been shown to mediate the

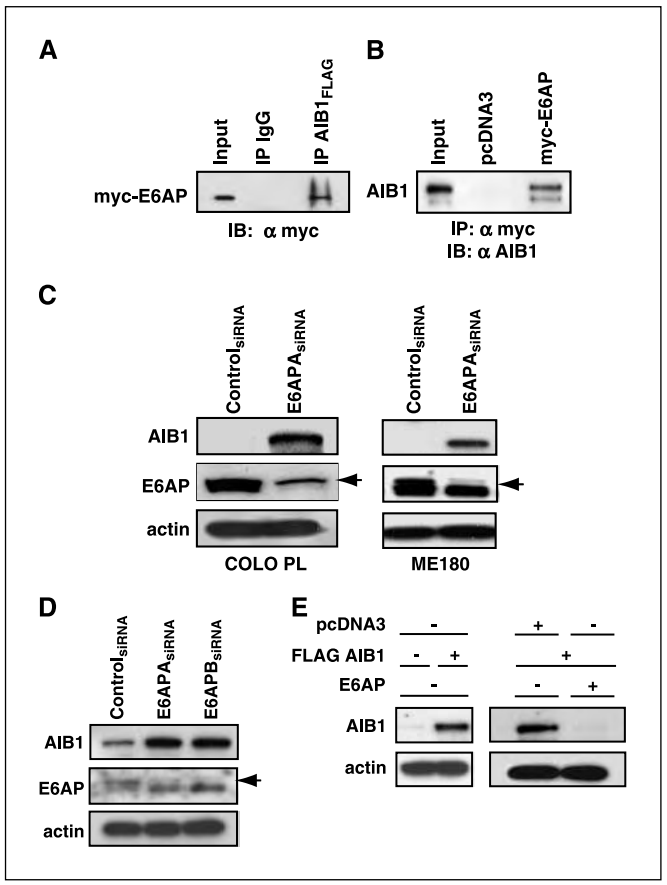

Figure 4. AIB1 interacts with E6AP *in vitro* and *in vivo* and E6AP is required for the proteasomal degradation of AIB1. *A,* AIB1 interacts with E6AP *in vivo*. HEK293T cells were transfected, in the presence of ALLN, with equal amounts of FLAG-tagged AIB1 and myc-tagged E6AP. Cells were harvested, and whole-cell extract was made with RIPA, divided into two aliquots, and immunoprecipitated with either anti-FLAG antibody or mouse IgG. Precipitated proteins were analyzed by Western blot and probed with anti-Myc antibody. *B,* E6AP interacts with endogenous AIB1. MCF-7 cells were transfected with pcDNA3 vector or myc-tagged E6AP, serum starved in the presence of ALLN, and immunoprecipitated with anti-Myc antibody. Precipitated proteins were analyzed by Western blot and probed with anti-AIB1 antibody. *C,* suppression of E6AP protein levels by siRNA leads to AIB1 protein stabilization. COLO 357 PL or ME-180 cells, grown to high density, were treated with control siRNA or E6APA siRNA (32), and cell lysates were analyzed by Western blot for AIB1 or E6AP. *Arrow,* the upper band (MW, 100 kDa) in the E6AP Western analysis is E6AP. The lower band detected with the E6AP antibody is nonspecific. *D,* suppression of E6AP protein levels can be achieved by two different E6AP siRNAs and leads to AIB1 protein stabilization. ME180 cells were treated with control siRNA, E6APA siRNA, or E6APB siRNA (32), and cell lysates were analyzed by Western blot. *E,* overexpression of E6AP in HEK293T cells causes reduced expression of AIB1. *Right,* cells were cotransfected with both FLAG-tagged AIB1 and a pcDNA3 vector control or an E6AP expression vector. After serum deprivation, whole-cell lysates were prepared, and FLAG-AIB1 levels were observed by Western blot with an AIB1 antibody (*right*). *Left,* comparison of endogenous AIB1 levels to AIB1 levels after transfection of FLAG-AIB1 in HEK293T cells.

E6-dependent proteasomal degradation of hScrib and E6TP1 (41, 42) and the E6-independent proteasomal degradation of Blk and HHR23A (43, 44). It had been described previously that AIB1 and E6AP form a complex (40). However, it had not been shown that E6AP plays a direct role in the degradation of AIB1 nor that the direct interaction of E6AP with the COOH terminus of AIB1 is required for degradation. The published literature to date suggests that E6AP has a dual role as both a steroid receptor coactivator and an ubiquitin ligase (39). Our results here suggest the possibility that E6AP may undergo a switch between these two functions dependent on cellular conditions. Under certain circumstances, it may be that the E6AP, in complex with AIB1, serves a coactivation function (39), whereas, in other cellular conditions, such as serum deprivation, the E6AP-AIB1 complex serves to ubiquitylate and target AIB1 for proteasomal degradation. In our model (Fig. 6), this switch of function in E6AP would be dependent on cellular signaling pathways, which are activated by changes in the cellular milieu.

Our data suggest that the challenge of cells with serum deprivation or high cell density initiate an early change in a signaling pathway that rapidly rids the cell of a pro-proliferative coactivator. The nature of the signaling pathways involved in the activation of AIB1 degradation is not known. In preliminary studies, we found that conditioned medium from cells grown at high density induce degradation of AIB1 when transferred to other cells (data not

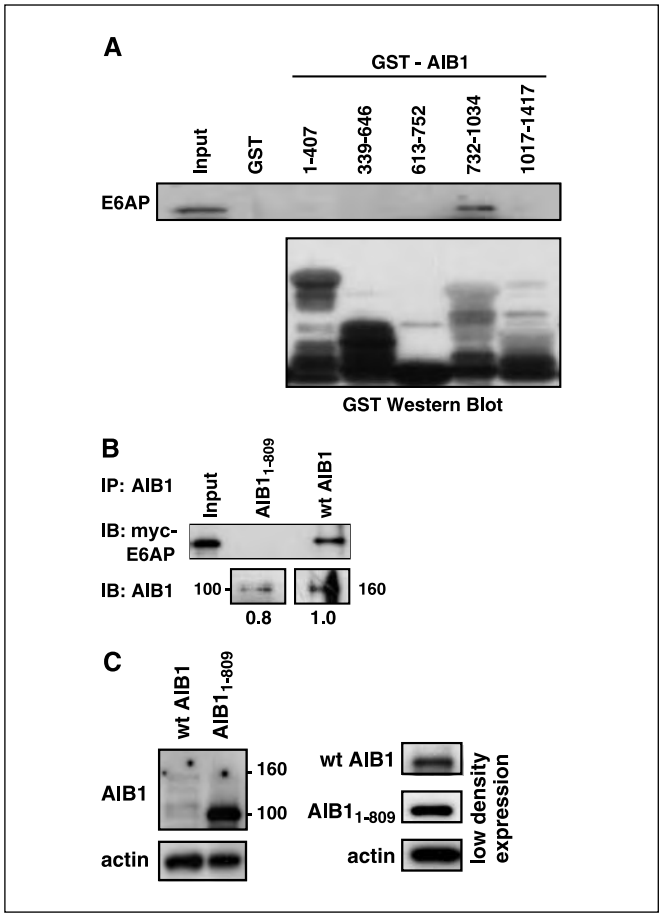

Figure 5. *A,* GST pull-down assay shows E6AP interacts with AIB1 *in vitro* and that the interaction domain involves residues within the COOH terminus of AIB1. A series of protein fragments of AIB1 fused to GST were produced in *Escherichia Coli* and incubated with MCF-7 cell extracts that contain E6AP. After elution from the glutathione beads, the amount of E6AP bound to each fragment was determined by a Western blot probed with E6AP antibody. To determine total expressed GST protein, equal aliquots of purified GST fragments were resolved on a gel, transferred to a PVDF membrane, and analyzed by Western blot with a GST antibody. *B,* AIB1 full length, but not an AIB1 1 to 809 truncation mutants, is able to coimmunoprecipitate with myc-tagged E6AP. *Bottom,* the total amount of transfected protein was quantitated by scanning densitometry. Relative scanning levels of the bands. *C,* COOH terminus truncation mutant of AIB1 (residues 1-809) is resistant to proteasomal degradation compared with full-length AIB1. Expression vectors for full-length AIB1 or AIB1 truncation mutants (1-809) were transfected into HEK 293T cells plated at high cell density. *Left,* cell lysates were harvested 24 hours after transfection and AIB1 protein levels were analyzed by Western blot. *Right,* initial transfected levels at low density of full-length AIB1 and the 1-809 truncation mutant were similar as assessed by Western blot analysis of whole-cell extracts.

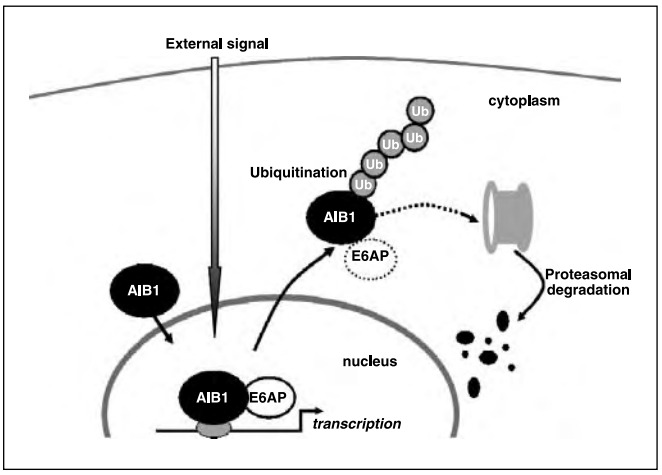

Figure 6. A model for the regulation of AIB1 degradation involving E6AP. Either pretranslocation or post-translocation into the nucleus, AIB1 forms a complex either directly through its COOH terminus with E6AP or with an intermediary E6AP-regulated protein. Ultimately, an E6AP-AIB1 complex potentiates transcription in the nucleus (36). When the cell is exposed to serum deprivation or cells are at high density, a cellular signaling pathway is rapidly activated that causes AIB1/E6AP to disengage from the transcription complex and exit the nucleus. In the cytoplasm, the AIB1 complex is then targeted for ubiquitylation, using the ubiquitin ligase function of E6AP, and subsequent degradation by the proteasome.

shown), suggesting that these events may be triggered by autocrine growth factor(s) that accumulates under these conditions. Many solid tumors have been reported to express high levels of AIB1 protein relative to the respective normal tissue (1–6), and the lack of these regulatory factor(s) in the tumor tissue may be one explanation of the up-regulation of AIB1 protein *in vivo*. Alternatively, it has been reported previously that the expression of E6AP is down-regulated in advanced-stage carcinomas of the breast (45). In light of our results, which implicate E6AP as the ubiquitin ligase involved in the degradation of AIB1, the down-regulation of E6AP in solid tumors may provide an alternate mechanistic explanation for the up-regulation of AIB1 protein in tumors especially where there is no amplification of the *AIB1* gene. Interestingly, down-regulation of E6AP has been associated with up-regulation of the estrogen receptor in breast carcinomas and the androgen receptor in prostate carcinomas (45). Overexpression of AIB1 has been reported in both of these tumor types (2, 6) and it will be interesting to determine if the down-regulation of E6AP is also associated with up-regulation of AIB1 in other human cancers. AIB1 protein is also overexpressed during progression of pancreatic cancer and in advanced pancreatic adenocarcinoma (3). Pancreatic adenocarcinoma is not a hormone-dependent tumor but is highly dependent on up-regulated IGF and epidermal growth factor signaling. AIB1 is involved in phenotypic and signaling changes induced by both of these signaling pathways (17, 18, 46). In addition, AIB1 is a coactivator for transcription factors, such as nuclear factor-κB (47), STAT6 (48), and transcriptional enhancer factors (49). It will be particularly interesting to see if there is a correlation between low levels of E6AP in pancreatic cancer and the overexpression of AIB1 and these other factors in the progression of pancreatic adenocarcinoma.

The model in Fig. 6 takes into account the observations presented above and the *in vivo* observation that AIB1 is a potent pro-proliferative, oncogenic molecule in animal models (11, 12) whose cellular levels would normally need to be tightly controlled at the transcriptional as well as translational level. Here, we show, in epithelial cell lines, that challenging with serum deprivation or growth at high cell density induces the regulated proteasomal degradation of AIB1. In addition, this process, which requires nuclear export, involves E6AP, which can interact directly with AIB1. Of note is that the reduction in AIB1 protein expression in response to serum withdrawal was not directly correlated with a change to a particular phase of the cell cycle per se because the percentage of cells in G_2-S phase versus the G_0-G_1 phase after 10 to 12 hours of serum withdrawal did not significantly change in COLO-PL cells (data not shown).

A major question is whether the E6AP-mediated degradation of AIB1 is a direct or indirect effect. To answer this question, we have conducted an *in vitro* ubiquitylation assay (29) with recombinant GST-E6AP and *in vitro* transcribed translated AIB1. In these preliminary experiments, we were able to observe binding between AIB1 and GST-E6AP but were unable to detect ubiquitylated species of AIB1 (data not shown). This result could imply that E6AP-mediated degradation of AIB1 requires the presence of an intermediary protein. Interestingly, the peptidyl-prolyl isomerase Pin1 has been shown recently to promote the degradation of AIB1 (50). Whether Pin1 is involved in and required for E6AP-mediated degradation of AIB1 is an interesting question and has yet to be determined.

Finally, in our model (Fig. 6), we postulate that serum withdrawal or high cell density activates a cellular signaling pathway that initiates AIB1 degradation. In preliminary studies to determine potential signaling molecules involved in this pathway, we have tested constructs with mutations of five previously identified serine phosphorylation sites in AIB1 that are the recipients of cellular signals (e.g., phosphatidylinositol 3-kinase and Jun kinase; ref. 51). However, none of these AIB1 serine phosphorylation mutants were resistant to induced degradation (data not shown). In contrast, our data indicate that a mutant missing a large portion of the COOH terminus is resistant to proteasomal degradation. This suggests that novel regulatory pathways might be involved in initiating the degradation of AIB1, possibly involving post-translational modifications in the COOH terminus of AIB1.

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